Effects of Engineering Complementary Charged Residues into the Hydrophobic Subunit Interface of Tyrosyl-tRNA Synthetase†

Appendix: Kinetic Analysis of Dimeric Enzymes That Reversibly Dissociate into Inactive Subunits

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Received August 5, 1986; Revised Manuscript Received December 8, 1986

ABSTRACT: Wild-type tyrosyl-tRNA synthetase (TyrTS) from *Bacillus stearothermophilus* is a symmetrical dimer. Four different heterodimeric enzymes have been produced by site-directed mutagenesis at the subunit interface so that the monomers are linked by a potential salt bridge in a hydrophobic environment. The two Phe-164 residues of wild-type TyrTS are on the axis of symmetry and interact in a hydrophobic region of the subunit interface. Mutation of Phe-164 to aspartate or glutamate in full-length TyrTS and to lysine or arginine in an active truncated enzyme (ΔTyrTS) induces reversible dissociation of the enzyme into inactive monomers. Mixing mutants in equimolar amounts produces four different heterodimers: TyrTS(Asp-164)-ΔTyrTS(Lys-164); TyrTS(Asp-164)-ΔTyrTS(Arg-164); TyrTS(Glu-164)-ΔTyrTS(Lys-164); TyrTS(Glu-164)-ΔTyrTS(Arg-164). A general method is derived for analyzing the kinetics of dimeric enzymes that reversibly dissociate into inactive subunits. Application to mutants of TyrTS allows estimation of dissociation constants (K_d values) for the dimers. At pH 7.8, the heterodimers have K_d values of 6–14 μM, whereas for homodimers K_d = 120–4000 μM. These values decrease to about 30 μM for homodimers of TyrTS(Asp-164), TyrTS(Glu-164), and ΔTyrTS(Lys-164) when the pH favors uncharged forms of the side chains at position 164. Each of the four salt bridges engineered into the hydrophobic subunit interface of TyrTS appears, therefore, to be weak. These engineered salt bridges may be compared with naturally occurring ones. In the latter, there are complementary interactions between the charges in the salt bridge with polar groups in the protein. The potential salt bridges engineered into TyrTS were not designed to be stabilized in this way. They are consequently weak but are sufficient to direct specificity in dimerization.

Tyrosyl-tRNA synthetase (TyrTS)[1] from *Bacillus stearothermophilus* is composed of two identical subunits each of M_r 47 300 (Winter et al., 1983) and each having a complete active site. The enzyme displays half of the sites activity in that only one active site appears functional per dimer (Fersht et al., 1975). Each monomer has two domains. The subunits interact through hydrophobic regions on the amino-terminal domains, and the carboxyl-terminal domains are not involved in contacts between monomers (Blow & Brick, 1985). The enzyme catalyzes aminoacylation of tRNA as a two-step reaction (eq 1 and 2).

$$E + ATP + Tyr \rightleftharpoons E\cdot Tyr\text{-}AMP + PP_i \quad (1)$$

$$E\cdot Tyr\text{-}AMP + tRNA \rightarrow E + Tyr\text{-}tRNA + AMP \quad (2)$$

Deletion of the carboxyl-terminal domains (residues 321–419) of TyrTS produces ΔTyrTS (M_r 2 × 36 300), abolishing the binding of tRNA but leaving the kinetics of formation of tyrosyl adenylate and pyrophosphate exchange (eq 1) virtually unaffected (Waye et al., 1983; Wells & Fersht, 1985).

The two Phe-164 side chains in wild-type enzyme interact in a hydrophobic region at the subunit interface and lie on the axis of symmetry (Blow & Brick, 1985). Mutation of TyrTS(Phe-164) to TyrTS(Asp-164) causes reversible dissociation of the dimer, especially at high pH where there is a tendency to ionize at position 164 (Jones et al., 1985). Phe-164 has also been mutated to lysine in ΔTyrTS to induce dissociation analogous to that of TyrTS(Asp-164) but with the opposite pH dependence (Ward et al., 1986). The monomeric enzymes appear to be inactive and unable to bind tyrosine. Studies using gel-filtration chromatography and kinetic analysis showed that mixing monomeric TyrTS(Asp-164) with monomeric ΔTyrTS(Lys-164) produces active heterodimers (Ward et al., 1986).

Modeling by computer graphics indicated that a heterodimer of TyrTS(Asp-164) with ΔTyrTS(Lys-164) could have the carboxylate and ϵ-NH_3^+ situated at the correct distance and orientation for the formation of a salt bridge between the two (or a hydrogen bond between the carboxyl and ϵ-NH_2 if proton transfer occurs). As it is possible that other combinations of acidic and basic residues may make better interactions, we have now constructed four different heterodimers. Phe-164 has been mutated to glutamate in full-length TyrTS and to arginine in ΔTyrTS. Heterodimers are produced by combining TyrTS carrying an acidic residue at position 164 with truncated enzyme having a basic side chain at position 164. We derive methods for determining the dissociation constants of dimeric enzymes that are in equilibrium with inactive monomers. These methods are applied to TyrTS mutants, allowing investigation of the effects of introducing complementary charged side chains into a hydrophobic environment.

Experimental Procedures

Materials

Reagents were purchased from Sigma (London), Cambridge Biotechnology Ltd., and Amersham International.

Construction of Genes for Mutant Enzymes. The change TyrTS(Phe-164) to TyrTS(Glu-164) was made by site-directed

† This work was supported by the Medical Research Council of the U.K.

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[1] Abbreviations: TyrTS, tyrosyl-tRNA synthetase; ΔTyrTS, truncated tyrosyl-tRNA synthetase [see Waye et al. (1983)]; Tris, tris(hydroxymethyl)aminomethane; Bistris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.

0006-2960/87/0426-4131$01.50/0

mutagenesis (Winter et al., 1982) with an oligonucleotide of sequence 5′AACTCGGTT*T*C*TGAAAT3′ (* = mismatched base). The ΔTyrTS(Phe-164) gene (Waye et al., 1983) was mutated to code for Arg-164 by the same method with an oligonucleotide of sequence 5′AACTCGGTAC*G*TGAAAT3′. The mutations were confirmed by dideoxy-DNA sequencing (Sanger et al., 1977). Construction of the genes for TyrTS(Asp-164) and ΔTyrTS-(Lys-164) has been described previously (Jones et al., 1985; Ward et al., 1986).

Expression and Purification of Enzymes. All enzymes were expressed and prepared as described by Lowe et al. (1985). Any TyrTS activity from the *Escherichia coli* host was destroyed by incubation at 56 °C for 30 min. Each preparation was homogeneous on $NaDodSO_4$–polyacrylamide gel electrophoresis.

Methods

Kinetic Procedures. All experiments were performed at 25 °C and at an ionic strength of 0.12 M. The following final concentrations of buffers were used: pH 6.0, 128 mM Bis-tris-HCl; pH 7.8, 144 mM Tris-HCl; pH 9.3, 60.4 mM glycine–NaOH. Each buffer contained 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride.

Active site titration by filtration through nitrocellulose disks is normally used to measure the concentration of TyrTS (Wilkinson et al., 1983). The assay consists of measuring accumulation of the stable enzyme-bound tyrosyl adenylate complex in the presence of pyrophosphatase (eq 1). This procedure can underestimate concentrations of mutants at position 164 since these enzymes dissociate into inactive monomers (Ward et al., 1986). Concentration of enzyme was therefore determined spectrophotometrically (based on A_{280} = 1.05 for a 1 mg cm^{-3} solution of TyrTS and A_{280} = 1.12 for a 1 mg cm^{-3} solution of ΔTyrTS) and is quoted in terms of dimers throughout this work. This method is reliable, since when used to titrate two mutants to form a heterodimer of TyrTS(Asp-164) with ΔTyrTS(Lys-164), the expected 1 mol of tyrosyl adenylate/mol of dimer is found (Ward et al., 1986). The pyrophosphate exchange procedure of Calendar and Berg (1966) was used to assay activation of tyrosine.

Computer Graphics. Model-building on an Evans and Sutherland PS300 was performed with the program FRODO (Jones, 1982). Coordinates from analysis of X-ray diffraction by crystals of TyrTS(Phe-164) and ΔTyrTS(Phe-164) were kindly supplied by P. Brick and D. M. Blow (Department of Physics, Imperial College).

RESULTS

Kinetics of Pyrophosphate Exchange. Replacement of Phe-164 in TyrTS by aspartate or lysine induces reversible dissociation of the enzyme into inactive monomers (Ward et al., 1986). High concentrations of tyrosine or ATP favor dimerization of the mutant enzymes since monomers appear unable to bind these substrates (see Appendix). The contribution of each substrate to stabilization of dimer is a function of $[S]/K_s$ (where K_s is the dissociation constant for substrate from dimeric enzyme).

The kinetic properties of mutant TyrTS were studied at three pH values: 6.0, 7.8, and 9.3. Pyrophosphate exchange kinetics were characterized by measurement of k_{cat} and K'_M (the apparent value of K_M for the particular conditions, see below). Mutants and wild-type enzyme displayed similar values of k_{cat} (Table I) under conditions where high concentrations of substrates favor dimerization. The relationship between $v/[E]_t$ and $v/[S][E]_t$ is not linear when mutants dissociate (see Appendix). The Michaelis constant, therefore, cannot be measured as the gradient of the Eadie plot. To avoid this problem, the apparent Michaelis constant (represented by the symbol K'_M) was measured as the concentration of substrate giving a rate equal to $V_{max}/2$ for wild-type enzyme. V_{max} for the wild-type enzyme is used because the value is often difficult to measure for mutant TyrTS (see Appendix); therefore, this approach utilizes the observation that mutation does not appear to change the V_{max} (see below).

The observed kinetics of pyrophosphate exchange suggest that TyrTS(Glu-164) and ΔTyrTS(Arg-164) each display pH-dependent dissociation in an analogous way to TyrTS-(Asp-164) and ΔTyrTS(Lys-164), respectively. Mutation does not appear to have a large affect on ATP dependence at high concentrations of tyrosine (Table I). This is probably because the ratio of $[S]/K_s$ is much greater for tyrosine than it is for ATP, so that tyrosine is principally responsible for stabilization of dimeric enzyme. In the presence of a low concentration of ATP, every mutant homodimer has a greatly elevated value of K'_M for tyrosine at pH 7.8 compared to that of wild-type enzyme or ΔTyrTS(Phe-164) (Table I). This is probably because tyrosine is again mainly responsible for stabilization of dimers. Mutant homodimers behave analogously to each other in displaying higher values of K'_M for tyrosine when the pH increasingly favors ionization at position 164 (Table I). At low pH, TyrTS(Asp-164) and TyrTS(Glu-164) start to resemble wild-type enzyme; conversely the K'_M values of ΔTyrTS(Lys-164) and ΔTyrTS(Arg-164) are lower at high pH.

ΔTyrTS(Arg-164) has a very high K'_M for tyrosine at every pH (Table I). This could be due to dissociation or a decreased affinity of binding by dimeric enzyme. Gel filtration using fast protein liquid chromatography [see Jones et al. (1985) and Ward et al. (1986)] showed that the enzyme is predominantly monomeric at pH 7.8, even at high concentration (18 μM) in the presence of 250 μM tyrosine, 2 mM ATP, 2 mM pyrophosphate, and 12 mM $MgCl_2$. This indicates that, under the conditions used for the pyrophosphate exchange assay, dissociation is largely responsible for the kinetic properties of ΔTyrTS(Arg-164). This conclusion is supported by the observation that ΔTyrTS(Arg-164) appears to function normally in heterodimers containing this mutation (see below).

An equimolar mixture of ΔTyrTS(Lys-164) with TyrTS-(Asp-164) or TyrTS(Glu-164) exhibits a K'_M for tyrosine that is very much smaller than that for the parent homodimers under similar conditions (Table I). The value for the heterodimer of TyrTS(Asp-164) with ΔTyrTS(Lys-164) is almost identical with that of wild-type enzyme. Combination of TyrTS(Asp-164) or TyrTS(Glu-164) with equimolar ΔTyrTS(Arg-164) produces a less marked decrease in K'_M for tyrosine. However, the dissociation constants (Table II) indicate that heterodimers are favored over homodimers under these conditions. Thus, heterodimers predominate in each of the mixtures so that experimental data can be used *directly* to compare heterodimeric and homodimeric enzymes. Heterodimeric enzymes were not studied at pH 6.0, nor at pH 9.3, because formation of parent homodimers would be favoured so that data obtained would not be interpretable.

Mutant Dimers Resemble Wild-Type TyrTS When Association Is Favored. In order to analyze the kinetics of mutants at position 164, it is assumed that they and wild-type enzyme are identical in all rate and binding constants except K_d. This assumption is reasonable on account of the following: (a) mutants and wild-type enzyme have similar values of k_{cat}

Table I: Substrate Dependence of Pyrophosphate Exchange Kinetics[a]

enzyme	Tyr dependence K'_M (μM)	Tyr dependence k_{cat} (s^{-1})	ATP dependence K'_M (mM)	ATP dependence k_{cat} (s^{-1})
pH 6.0				
wild type	1.9	14	0.92	12
Δ(321–419)	2.1	15	1.1	13
TyrTS(Asp-164)[b,d]	6.2	11	0.91	10
TyrTS(Glu-164)	37	12	2.2	14
ΔTyrTS(Lys-164)[c]	>20 000			
ΔTyrTS(Arg-164)[c]	>20 000			
pH 7.8				
wild type	2.4	9.5	1.2	9.6
Δ(321–419)	2.4	8.6	1.2	9.7
TyrTS(Asp-164)[d]	270	9.0	2.1	10
TyrTS(Glu-164)	380	9.4	1.2	9.6
ΔTyrTS(Lys-164)	160	10	1.5	9.3
ΔTyrTS(Arg-164)[c]	>5 000			
TyrTS(Asp-164) + ΔTyrTS(Lys-164)	2.3	10	0.91	10
TyrTS(Asp-164) + ΔTyrTS(Arg-164)	290	7.7	1.1	9.9
TyrTS(Glu-164) + ΔTyrTS(Lys-164)	41	9.0	1.6	8.4
TyrTS(Glu-164) + ΔTyrTS(Arg-164)	240	8.1	1.3	9.1
pH 9.3				
wild type	2.4	12	1.2	10
Δ(321–419)	2.5	10	1.2	9.1
TyrTS(Asp-164)[c]	>6 000			
TyrTS(Glu-164)[c]	>20 000			
ΔTyrTS(Lys-164)	49	13	1.0	11
ΔTyrTS(Arg-164)[c]	>5 000			

[a] Experimental conditions are given under Methods. Pyrophosphate was at 2 mM, and $MgCl_2$ was maintained at 10 mM greater concentration than ATP. Dependence on tyrosine was measured at 2 mM ATP, and dependence on ATP was measured at 250 μM tyrosine. Δ(321–419) is truncated TyrTS (Waye et al., 1983). Enzyme concentrations were as follows: wild type, 0.20 μM; Δ(321–419), 0.32 μM; TyrTS(Asp-164), 1.0 μM; TyrTS(Glu-164), 0.95 μM; ΔTyrTS(Lys-164), 3.2 μM; ΔTyrTS(Arg-164), 1.8 μM; TyrTS(Asp-164) + ΔTyrTS(Lys-164), 0.50 μM each; TyrTS(Asp-164) + ΔTyrTS(Arg-164), 0.50 μM each; TyrTS(Glu-164) + ΔTyrTS(Lys-164), 0.47 μM each; TyrTS(Glu-164) + ΔTyrTS(Arg-164), 0.48 μM each. Values of k_{cat} were obtained by extrapolation to infinite concentration of second substrate assuming Michaelis–Menten kinetics. The value of K'_M was measured as the concentration of substrate giving a rate equal to $V_{max}/2$ for wild-type enzyme (see Results). [b] [Tyr] = 50 μM. [c] K'_M for tyrosine was greater than the solubility of the substrate. The value of K'_M was estimated by linear extrapolation from the data obtained with the maximum practical concentration of tyrosine ($\simeq$1000 μM) to the data for V_{max} for wild-type enzyme, assuming that dependence upon ATP was not changed by mutation at position 164. The values of K'_M and k_{cat} for ATP could not be calculated because the tyrosine-dependence could not be measured. [d] Calculated from the same data as Jones et al. (1985).

Table II: Dissociation Constants and Free Energy Changes for Dimerization of TyrTS Mutants[a]

enzyme	K_d (μM)[b] method I	K_d (μM)[b] method II	ΔG_a (kJ mol^{-1})
pH 6.0			
TyrTS(Asp-164)	c	31	−25.7
TyrTS(Glu-164)	12	34	−25.5
ΔTyrTS(Lys-164)	>20 000	50 000	−7.2
ΔTyrTS(Arg-164)	>12 000	40 000	−8.2
pH 7.8			
TyrTS(Asp-164)	76	120	−22.3
TyrTS(Glu-164)	98	150	−21.8
ΔTyrTS(Lys-164)	140	190	−21.2
ΔTyrTS(Arg-164)	>2 000	4 000	−13.7
TyrTS(Asp-164) + ΔTyrTS(Lys-164)	c	6.1	−29.8
TyrTS(Asp-164) + ΔTyrTS(Arg-164)	20	14	−27.7
TyrTS(Glu-164) + ΔTyrTS(Lys-164)	2.4	9	−28.8
TyrTS(Glu-164) + ΔTyrTS(Arg-164)	15	13	−27.9
pH 9.3			
TyrTS(Asp-164)	>2 000	4 000	−13.5
TyrTS(Glu-164)	>6 000	10 000	−10.7
ΔTyrTS(Lys-164)	17	27	−26.1
ΔTyrTS(Arg-164)	>4 000	5 000	−13.4

[a] Values were calculated from pyrophosphate exchange data. [b] Method I: Determined from K'_M for tyrosine. Method II: Mean value calculated from rate assays with varied concentration of enzyme. Replicates agreed to ±10%. ΔG_a was the free-energy change on association of subunits under standard thermodynamic conditions of 1 M enzyme in the absence of substrates. This was calculated as $\Delta G_a = RT \ln K_d$, taking the value of K_d from method II. [c] $[Tyr]/K_t < 5[ATP]/K_a$ when $[Tyr] = K'_M$ so that K_d could not be calculated.

(Table I), showing that the changes have no overall effect on catalysis; (b) the K'_M for ATP is not drastically altered by mutation (Table I), which therefore has no significant effect on affinity for the substrate; (c) although mutations change the values of K'_M for tyrosine, this is mainly because of dissociation into subunits that have little affinity for the substrate. For TyrTS(Asp-164) and ΔTyrTS(Lys-164), the K'_M for tyrosine varies with pH in the same manner as does dimerization [Table I and Ward et al. (1986)]. When dimerization is favored, such as for TyrTS(Asp-164) at pH 6.0, the dissociation constant of tyrosine (K_t) is almost unchanged from that for the wild type (Jones et al., 1985), demonstrating directly that the tyrosine-binding site is not perturbed. K_t cannot be measured for other mutants or conditions because of dissociation of the enzymes. But a heterodimer of TyrTS(Asp-164) with ΔTyrTS(Lys-164) exhibits a K'_M for tyrosine at pH 7.8 that is very similar to that of wild-type enzyme (Table I), showing that either binding is unchanged in each mutant or the two changes mutually compensate.

Determination of Dissociation Constants for TyrTS Mutants. Equations are derived in the Appendix to analyze the pyrophosphate exchange kinetics of TyrTS mutants. The basis of the analysis is that substrate binds only to dimeric enzyme; therefore, dissociation of the enzyme decreases affinity for the ligands causing an increased value of apparent K_M (which is represented by the symbol K'_M). The measured value of K'_M is related to the proportion of dimer that has dissociated and can be used to calculate the dissociation constant (K_d) of the dimeric enzyme.

The value of K_d can be measured by comparing the pyrophosphate exchange kinetics of the wild type (which does not dissociate to a detectable extent) with those of the mutants. Data are collected at low ATP concentration so that tyrosine is principally responsible for stabilization of the active dimers. Two approaches are used to estimate K_d (see Appendix). In method I, K_d is calculated from the value of K'_M for tyrosine, whereas in method II the value of K_d is determined from the rate of reaction at different concentrations of enzyme. Method II is more accurate than method I for technical reasons (see Appendix).

(a) Dissociation Constants of Mutant Homodimers. Each mutant homodimer displays pH-dependent association. The value of K_d increases with the tendency to ionize at position 164 (Table II), demonstrating that homodimers do not contain two charged residues at this position.

The observed value of K_d at any pH is a function of the microscopic dissociation constant of the dimer and the ionization constant of the groups at position 164. The limited pH range of the pyrophosphate exchange assay prevents measurement of the value of K_d when there is no charge at this

position. The ionization constants of the residues at position 164 can, therefore, only be estimated, and the values do not seem to be highly perturbed. This would be expected because the side chains are at the surface of the monomers. The dimers may have zero charge or one charge at position 164. The change in protonation of the residue at this position on dimerization may be deduced from the pH dependence of K_d. At pH values above the pK_a of Asp-164 or Glu-164, a plot of log K_d against pH should give a gradient equal to the number of protons taken up. Conversely, the same theory should hold for the number of protons lost upon dimerization by Lys-164 or Arg-164 when the pH is below the pK_a. The gradient most closely approximates to one in each case, suggesting that there is a single charge at position 164 of the homodimers.

The effect of extent of ionization on the observed value of K_d is seen by comparing ΔTyrTS(Lys-164) to ΔTyrTS(Arg-164) (Table II). The pK_a values of Lys-164 and Arg-164 are likely to be approximately 10 and 12, respectively. Thus at high pH, where Arg-164 is ionized to a greater extent than Lys-164, ΔTyrTS(Arg-164) displays a higher value of K_d than does ΔTyrTS(Lys-164). However, each dimer has a similar value of K_d at low pH where both residues are essentially fully ionized.

The size of the side chain at position 164 also appears to influence subunit association in homodimers. The four mutant side chains decrease in size in the order Arg > Lys > Glu > Asp. The effect of these changes may be seen by comparing the K_d values of the maximally ionized forms because each residue carries a single charge. Taking the values for pH 9.3 for the acidic mutants and the values for pH 6.0 for the basic mutants, then K_d decreases in the order Arg $\simeq$ Lys > Glu > Asp (Table II). The value of K_d therefore tends to change in parallel with the increase in size of the residue at position 164. This may result from steric effects, changed interactions with neighboring residues, or repulsion caused by the bringing of charges having the same polarity closer together.

(*b*) *Dissociation Constants of Heterodimers.* The values of K_d at pH 7.8 for the heterodimers are considerably lower than those for the homodimers (Table II). The polarity of the mutant side chain appears to have little effect on stability of the homodimers. However, the side chains of opposing polarity stabilize heterodimeric enzymes relative to the homodimers. This is consistent with qualitative analysis of dissociation by determining the values of apparent molecular mass (Ward et al., 1986).

The heterodimeric enzymes display a range of K_d values (Table II). Combination of TyrTS(Asp-164) with ΔTyrTS-(Lys-164) produces the most stable mutant enzyme. Heterodimers containing ΔTyrTS(Arg-164) are relatively unstable, reflecting the high K_d value of the parent homodimer at pH 7.8.

Discussion

Introduction of charged residues into the hydrophobic subunit interface of dimeric TyrTS induces reversible dissociation of the enzyme into inactive monomers. Mixtures of mutants with opposite charges at position 164, however, form relatively stable heterodimers. The dissociation constants of these mutants were calculated with a method for analyzing the kinetics of dimeric enzymes that are in equilibrium with inactive subunits. The analytical methods are derived in the Appendix.

Mutant Dimers Resemble Wild-Type Enzyme. Wild-type TyrTS and the mutant enzymes all show half of the sites activity. Although the mutations introduced in this study do weaken the association of subunits, they do not appear to change the functional interactions across the subunit interface or the overall integrity of the protein since mutant dimers appear to be fully active and show kinetic properties similar to the wild type when association is favored. Position 164 of TyrTS is, therefore, a suitable site to vary in studies of charged residues in a hydrophobic environment because the thermodynamic consequences of changes may be quantitated with enzyme kinetics.

Factors Affecting the Stability of Mutant Homodimers. In dimeric TyrTS, four phenylalanine residues form a hydrophobic box around the two interacting side chains at position 164 because of symmetry at the subunit interface. The two Phe-164 side chains are symmetrically positioned, as are those of Phe-167. The aromatic rings of the Phe-164 residues, together with those of the Phe-167 side chains, appear to be tetrahedrally arranged. This arrangement is flanked by the Phe-135 side chains. Computer graphics suggests that the closest contacts between Phe-164 and the other residues are 0.38 nm for Phe-135 and 0.39 nm for Phe-167.

Comparison of the free energies of association allows thermodynamic study of the effects of introducing charged residues into this hydrophobic box (Table II). Wild-type TyrTS dissociates to an immeasurably low extent. But, replacement of Phe-164 by fully ionized residues strongly favors dissociation of homodimers, mainly because of repulsion of charges having the same polarity and the high self-energy of charged groups in this environment. Homodimeric enzymes appear to be ionized at one of the two residues at position 164. Any charge on these side chains in mutant dimers is perhaps stabilized by water being trapped at this position in the subunit interface during the association of monomers to form dimers. Mutant dimers are much less stable than the wild type even when the two residues at position 164 have opposite polarity, which could attract the subunits to each other. The magnitude of these effects cannot be measured relative to wild-type enzyme because dissociation of native TyrTS has never been detected.

Stability of Heterodimers Results from Interactions between Complementary Residues. The four heterodimeric enzymes are stabilized, relative to the parent homodimers, by a salt bridge between the residues at position 164. There are contradictory theories about the strength of salt bridges in hydrophobic environments: interactions may be strong because of the low dielectric constant or weak due to the high self-energy of charged groups [see Warshel et al. (1984) and Honig and Hubbel (1984)]. Formation of heterodimeric enzymes could bring opposite charges close together and thus allow charge–charge interactions. However, residues at position 164 in the heterodimeric enzymes may not be ionized because of the local hydrophobicity. Thus, there is possibly a polar hydrogen bond, rather than a charge–charge interaction, that stabilizes the quaternary structure relative to that of mutant homodimers. Formation of each heterodimeric enzyme liberates only a little more free energy than does formation of homodimers (Table II), showing that each of these four salt bridges is weak. However, any hydrogen bonds engineered into TyrTS may not be optimal in length or angle and so may underestimate the potential strengths of such interactions. The contribution of ionization constants to free-energy changes on formation of heterodimers is not known because of uncertainty about the number of charges at position 164 in these enzymes. Quantitative measurement of the strength of these salt bridges is, therefore, not possible from these data.

At pH 7.8, the dissociation constants for heterodimers are much lower than those for the parent homodimers (Table II),

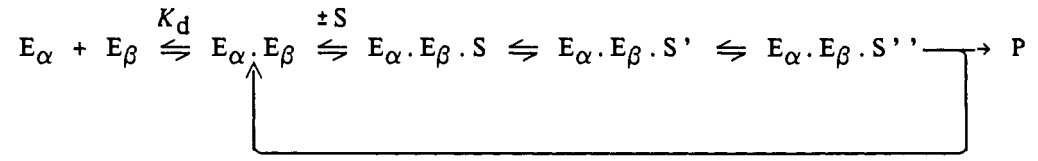

FIGURE 1: Reaction scheme for a dimeric enzyme that is in equilibrium with inactive subunits. This scheme is explained in the Appendix.

indicating that mixing a mutant having an acidic residue at position 164 with an enzyme carrying a basic side chain at this position favors formation of heterodimers. This shows that the interactions between the complementary residues are sufficient to direct specificity in subunit association.

Comparison with Naturally Occurring Buried Salt Bridges. The salt bridges engineered into TyrTS are crude because the complementary residues are designed to form favorable interactions just with each other. But, in naturally occurring buried salt bridges, there are generally additional interactions between the charged groups and permanent dipoles (hydrogen-bond donors and acceptors) in the protein so that all possible hydrogen bonds are formed (Warshel et al., 1984). The dipoles stabilize the charged groups in the salt bridge by lowering their self-energies. For example, the following buried salt bridges are all stabilized by a network of hydrogen bonds from the charged residues to nearby groups in the protein: that in chymotrypsin between Ile-16 and Asp-194 (Matthews et al., 1967), that between His-57 and Asp-102 in the "charge-relay system" of the same enzyme (Blow et al., 1969), and that between Arg-138 and Asp-194 in protease A from *Streptomyces griseus* (Brayer et al., 1978). The local environment of these is relatively hydrophilic. When salt bridges occur on the exterior of proteins, the charged residues are stabilized by hydrogen bonds to water molecules in the solvent. A numerical estimate of the stabilization energies of the neighboring dipoles may be made from experiments in which hydrogen-bond donors or acceptors have been deleted in an enzyme–substrate complex to leave partly desolvated charges: upward of 12 kJ mol^{-1} is lost (Fersht et al., 1985). The strengths of the crude salt bridges engineered into the tyrosyl-tRNA synthetase are accordingly much lower than those of naturally occurring salt bridges and greatly weaken association relative to wild-type enzyme. But, the complementary nature of interactions is sufficient to direct specificity in subunit association.

APPENDIX: KINETIC ANALYSIS OF DIMERIC ENZYMES THAT REVERSIBLY DISSOCIATE INTO INACTIVE SUBUNITS

Theoretical Basis for Method of Analysis. Substrate binds only to dimeric enzyme; therefore, dissociation of the enzyme decreases affinity for the ligands causing an increased value of observed K_M (which is represented by the symbol K'_M). The measured value of K'_M is related to the proportion of dimer that has dissociated and can be used to calculate the dissociation constant of the dimeric enzyme.

General Method for Kinetic Analysis of a Dimeric Enzyme Which Is in Equilibrium with Inactive Subunits. Inactive monomers, E_α and E_β, associate forming active dimer (see Figure 1). E_α and E_β are different in heterodimers, whereas they are identical in homodimers. Substrate binds only to dimeric enzyme, and then the reaction passes through intermediates, $E_\alpha{\cdot}E_\beta{\cdot}S'$ and $E_\alpha{\cdot}E_\beta{\cdot}S''$, finally forming and releasing product, P. The dimeric enzyme follows Michaelis–Menten kinetics when dissociation into subunits does not occur. The value of k_{cat} is not changed by dissociation if dimerization is not rate limiting. Mutants of TyrTS are readily analyzed by such a scheme because they can be preincubated with substrates and allowed to reach chemical equilibrium before addition of a trace of radioactive pyrophosphate to monitor rate of reaction. Let the total concentration of dimeric enzyme be $\sum[E_\alpha{\cdot}E_\beta]$; then

$$v = k_{cat}[S]\sum[E_\alpha{\cdot}E_\beta]/(K_M + [S]) \quad (A1)$$

Therefore

$$\sum[E_\alpha{\cdot}E_\beta] = v(K_M + [S])/k_{cat}[S] \quad (A2)$$

$\sum[E_\alpha{\cdot}E_\beta]$ may thus be estimated from the rate of reaction if the kinetic properties of the enzyme are known. These properties can often be determined at high enzyme concentration, when dimers predominate. K_M is the overall dissociation constant for all enzyme–substrate complexes (Fersht, 1985); that is

$$K_M = [E_\alpha{\cdot}E_\beta][S]/\sum[E_\alpha{\cdot}E_\beta{\cdot}S] \quad (A3)$$

where $\sum[E_\alpha{\cdot}E_\beta{\cdot}S]$ is the total concentration of all complexes between enzyme and substrate. K_M is an absolute value for the dimers and not an apparent value that is affected by the position of the equilibrium between monomers and dimers. $\sum[E_\alpha{\cdot}E_\beta{\cdot}S]$ is found by applying conservation of mass:

$$\sum[E_\alpha{\cdot}E_\beta{\cdot}S] = \sum[E_\alpha{\cdot}E_\beta] - [E_\alpha{\cdot}E_\beta] \quad (A4)$$

This expression can be substituted into eq A3 and then rearranged to give

$$[E_\alpha{\cdot}E_\beta] = \sum[E_\alpha{\cdot}E_\beta]K_M/(K_M + [S]) \quad (A5)$$

The total concentration of enzyme in terms of dimers when equimolar amounts of two types of subunit are mixed producing only heterodimers and monomers is

$$[E]_t = [E_\alpha]/2 + [E_\beta]/2 + \sum[E_\alpha{\cdot}E_\beta] \quad (A6)$$

Therefore

$$[E_\alpha] = [E_\beta] = [E]_t - \sum[E_\alpha{\cdot}E_\beta] \quad (A7)$$

The dissociation constant of subunits from a heterodimer is defined as

$$K_d = [E_\alpha][E_\beta]/[E_\alpha{\cdot}E_\beta] \quad (A8)$$

and its value may be found as follows. $\sum[E_\alpha{\cdot}E_\beta]$ can be calculated with eq A2, and the concentration of monomers may be determined with expression A7 if $[E]_t$ is known. [S] will not significantly change on addition of enzyme if $[S] \gg [E]_t$, so that $[E_\alpha{\cdot}E_\beta]$ can be calculated with eq A5. Values of $[E_\alpha]$, $[E_\beta]$, and $[E_\alpha{\cdot}E_\beta]$ may then be substituted into relationship A8.

The concentration of monomer formed on dissociation of homodimers is

$$[E_m] = [E_\alpha] + [E_\beta] = 2([E]_t - \sum[E_\alpha{\cdot}E_\beta]) \quad (A9)$$

Therefore

$$K_d = [E_m]^2/[E_\alpha{\cdot}E_\beta] \quad (A10)$$

The observed Michaelis constant, K'_M, is increased when a dimer dissociates because monomers cannot bind substrate. The high value of K'_M is, therefore, caused by an apparent increase in $[E_\alpha{\cdot}E_\beta]$. This increase is equal to the amount of dimer that dissociates. From eq A10, the concentration of monomer formed on dissociation of homodimeric enzyme is

$$[E_m] = (K_d[E_\alpha{\cdot}E_\beta])^{1/2} \quad (A11)$$

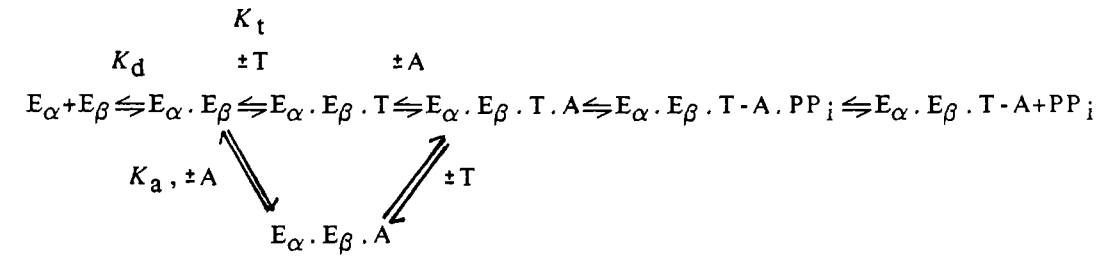

FIGURE 2: Scheme for pyrophosphate exchange by TyrTS (see Appendix). The symbols are as follows: T, tyrosine; A, ATP; T-A, tyrosyl adenylate; PP_i, pyrophosphate. The dissociation constants of the substrates from dimeric enzyme are K_t for tyrosine and K_a for ATP. K_t is 12 μM and K_a is 3.5 mM for wild-type TyrTS at pH 7.8 (Wells & Fersht, 1985; J. Knill-Jones and A. R. Fersht, unpublished data).

One mole of dimer yields 2 mol of monomer; therefore, the amount dissociating is $0.5[E_m]$. The apparent concentration of unliganded dimer is thus the actual concentration plus the amount that has dissociated:

$$=[E_\alpha \cdot E_\beta] + 0.5(K_d[E_\alpha \cdot E_\beta])^{1/2} \quad \text{(A12)}$$

By analogy to eq A3

$$K'_M = \{[E_\alpha \cdot E_\beta] + 0.5(K_d[E_\alpha \cdot E_\beta])^{1/2}\}[S]/\sum[E_\alpha \cdot E_\beta \cdot S] \quad \text{(A13)}$$

$$= \frac{[E_\alpha \cdot E_\beta][S]}{\sum[E_\alpha \cdot E_\beta \cdot S]} + \frac{(K_d[E_\alpha \cdot E_\beta])^{1/2}[S]}{2\sum[E_\alpha \cdot E_\beta \cdot S]} \quad \text{(A14)}$$

$$= K_M + (K_d[E_\alpha \cdot E_\beta])^{1/2}[S]/2\sum[E_\alpha \cdot E_\beta \cdot S] \quad \text{(A15)}$$

The value of K_d may be calculated from the measured K'_M by rearranging eq A15 so that when $[S] = K'_M$

$$K_d = 4\sum[E_\alpha \cdot E_\beta \cdot S]^2(K'_M - K_M)^2/K'^2_M[E_\alpha \cdot E_\beta] \quad \text{(A16)}$$

Values of $\sum[E_\alpha \cdot E_\beta \cdot S]$ and $[E_\alpha \cdot E_\beta]$ can be found from eq A4 and A5.

One mole of heterodimer dissociates to yield 1 mol of each type of monomer. The amount dissociating is given by rearranging eq A8:

$$[E_\alpha] = [E_\beta] = (K_d[E_\alpha \cdot E_\beta])^{1/2} \quad \text{(A17)}$$

The apparent concentration of unliganded dimer is thus the actual concentration plus the amount that has dissociated:

$$=[E_\alpha \cdot E_\beta] + (K_d[E_\alpha \cdot E_\beta])^{1/2} \quad \text{(A18)}$$

Note that this expression is different from that for a homodimeric enzyme (eq A12). The relationship between K_d and K'_M when $[S] = K'_M$ is therefore given by

$$K_d = \sum[E_\alpha \cdot E_\beta \cdot S]^2(K'_M - K_M)^2/K'^2_M[E_\alpha \cdot E_\beta] \quad \text{(A19)}$$

This equation for heterodimeric enzyme differs from that for homodimeric enzyme (eq A16) by a factor of 4.

Scheme for Pyrophosphate Exchange by TyrTS (Figure 2). The scheme is that of Wells and Fersht (1985) modified to include dissociation of the enzyme. Inactive monomers, E_α and E_β, are in equilibrium with active dimer, which has a dissociation constant of K_d. E_α and E_β are different in heterodimers, whereas they are identical in homodimers. Tyrosine or ATP bind to dimeric enzyme, which then associates with the second substrate. The reaction proceeds forming a complex of dimeric enzyme with tyrosyl adenylate and pyrophosphate, and finally, pyrophosphate dissociates.

In order to analyze the kinetics of mutants at position 164, it is assumed that they and wild-type enzyme are identical in all rate and binding constants except K_d (see Results). High concentrations of tyrosine or ATP favor dimeric forms of the mutant enzymes because monomers do not appear to bind these substrates (see below). The contribution of each substrate to stabilization of dimer is a function of $[Tyr]/K_t$ and $[ATP]/K_a$ (see Figure 2).

Kinetic Analysis of TyrTS Mutants Which Reversibly Dissociate. The equations derived above apply to enzymes having only one substrate. The kinetics of pyrophosphate exchange by mutant TyrTS may be analyzed in this way only if one substrate (say ATP) is fixed at a low concentration relative to its dissociation constant from the enzyme and the other substrate (say tyrosine) is varied but maintained at a high relative concentration (that is $[ATP]/K_a \ll [Tyr]/K_t$). Under these conditions, tyrosine is the only substrate that is significantly stabilizing dimeric enzyme. K_M for ATP is, therefore, unchanged from that of the wild type (Table I) and is given by (which is analogous to eq A3)

$$K_M = ([E_\alpha \cdot E_\beta] + [E_\alpha \cdot E_\beta \cdot T])[A]/\sum[E_\alpha \cdot E_\beta \cdot A] \quad \text{(A20)}$$

where $\sum[E_\alpha \cdot E_\beta \cdot A]$ is the total concentration of all complexes of enzyme and ATP and may be found by applying conservation of mass:

$$\sum[E_\alpha \cdot E_\beta \cdot A] = \sum[E_\alpha \cdot E_\beta] - ([E_\alpha \cdot E_\beta] + [E_\alpha \cdot E_\beta \cdot T]) \quad \text{(A21)}$$

The dissociation constant for tyrosine from $E_\alpha \cdot E_\beta \cdot T$ is defined as

$$K_t = [E_\alpha \cdot E_\beta][T]/[E_\alpha \cdot E_\beta \cdot T] \quad \text{(A22)}$$

Substituting eq A21 and A22 into eq A20 and rearranging gives

$$[E_\alpha \cdot E_\beta] = K_M\sum[E_\alpha \cdot E_\beta]/\{([A] + K_M)(1 + [T]/K_t)\} \quad \text{(A23)}$$

where K_M is that for ATP. $\sum[E_\alpha \cdot E_\beta]$ can be calculated from the measured rate of reaction with eq A2. The total concentration of all complexes between enzyme and tyrosine is given by

$$\sum[E_\alpha \cdot E_\beta \cdot T] = \sum[E_\alpha \cdot E_\beta] - ([E_\alpha \cdot E_\beta] + [E_\alpha \cdot E_\beta \cdot A]) \quad \text{(A24)}$$

which is analogous to expression A21. By comparison to relationship A22

$$[E_\alpha \cdot E_\beta \cdot A] = [E_\alpha \cdot E_\beta][A]/K_a \quad \text{(A25)}$$

Therefore

$$\sum[E_\alpha \cdot E_\beta \cdot T] = \sum[E_\alpha \cdot E_\beta] - [E_\alpha \cdot E_\beta](1 + [A]/K_a) \quad \text{(A26)}$$

Modification of eq A16 shows that

$$K_d = \frac{4\sum[E_\alpha \cdot E_\beta \cdot T]^2[K'_M - K_M]^2}{K'^2_M([E_\alpha \cdot E_\beta] + [E_\alpha \cdot E_\beta \cdot A])} \quad \text{(A27)}$$

for homodimeric enzyme, whereas the dissociation constant for heterodimeric enzyme is given by substitution into eq A19 so that

$$K_d = \frac{\sum[E_\alpha \cdot E_\beta \cdot T]^2[K'_M - K_M]^2}{K'^2_M([E_\alpha \cdot E_\beta] + [E_\alpha \cdot E_\beta \cdot A])} \quad \text{(A28)}$$

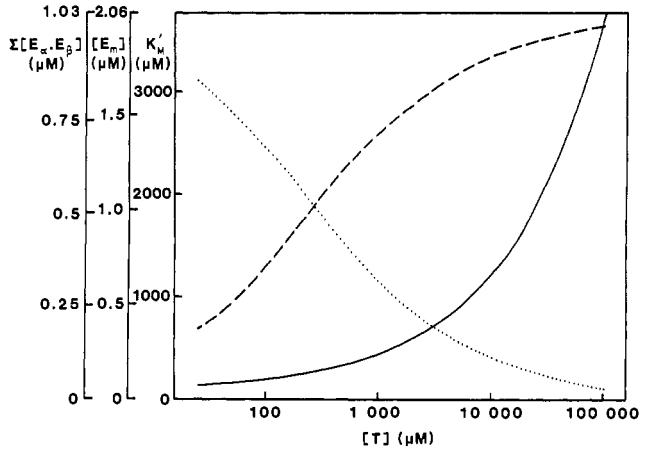

FIGURE 3: Predicted parameters for tyrosine dependence of pyrophosphate exchange by TyrTS(Asp-164) at pH 7.8. Conditions are as given in Table I. Data were predicted as follows: Equations A9 and A10 were substituted into expression A23, which was then rearranged and solved to give $[E_\alpha \cdot E_\beta]$ at different values of [Tyr]. $\sum[E_\alpha \cdot E_\beta]$ was calculated from $[E_\alpha \cdot E_\beta]$ with relationship A23, and $[E_m]$ was obtained from eq A9. K'_M for tyrosine was deduced by rearranging eq A27 and substituting in values from eq A23, A25, and A26. The value of K_d was taken as 120 μM. K'_M (—); $[E_m]$ (···); $\sum[E_\alpha \cdot E_\beta]$ (--).

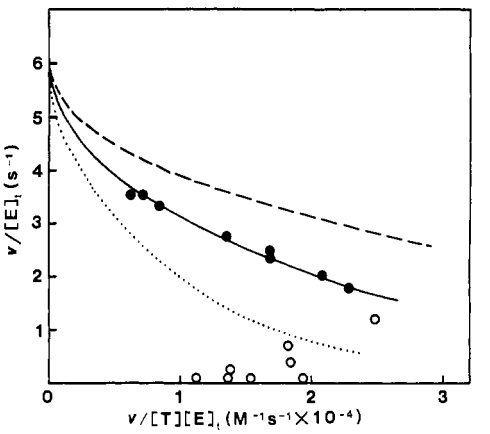

FIGURE 4: Eadie plot for tyrosine dependence of pyrophosphate exchange by TyrTS(Asp-164) at pH 7.8. Empirical data [from Jones et al. (1985)] that were obtained under the conditions given in Table I (● and ○). Using the same conditions, data were predicted as described in the legend to Figure 3 except values of $\sum[E_\alpha \cdot E_\beta]$ were substituted into eq A30 to calculate v. Predicted plots when K_d was taken as 120 (—), 62 (--), and 240 μM (···). Rate could be estimated only if $[Tyr]/K_t \gg [ATP]/K_a$; therefore, each plot was terminated when $[Tyr]/K_t < 5[ATP]/K_a$. For the data shown as (○), there was a disagreement between experimental data and those predicted using K_d = 120 μM.

where K_M and K'_M are values for dependency upon tyrosine. Values of $[E_\alpha \cdot E_\beta]$, $[E_\alpha \cdot E_\beta \cdot A]$, and $\sum[E_\alpha \cdot E_\beta \cdot T]$ can be determined from relationships A23, A25, and A26, respectively.

K_d can be estimated by two approaches. In the first (method I in Table II), the value is calculated from K'_M for tyrosine with eq A27 or A28 as described above. The second method (II in Table II) of determining K_d involves measurement of rate and then calculating $\sum[E_\alpha \cdot E_\beta]$ from the known properties of wild-type enzyme (expression A2). The concentration of monomeric enzyme, $[E_m]$, is estimated by subtracting $\sum[E_\alpha \cdot E_\beta]$ from $[E]_t$ (eq A9 for homodimer; eq A7 for heterodimer), and then $[E_\alpha \cdot E_\beta]$ is deduced from $\sum[E_\alpha \cdot E_\beta]$ (expression A23) so that the value of K_d can be determined (eq A10 for homodimer; eq A8 for heterodimer).

Measurement of K'_M is difficult because these enzymes give curved Eadie plots (see below). The value of K'_M is therefore taken as the concentration of substrate giving half-maximal rate for wild-type enzyme (see Results). The value cannot be measured accurately when K'_M is high because of the limitations imposed by the solubility of tyrosine. This prevents use of the substrate at above 1000 μM. The Eadie plots become very steep on approaching the $v/[E]_t$ axis, and often the curvature in this part of the plot cannot be measured because of the high concentration of tyrosine required. The enzymes often appear, therefore, to obey Michaelis–Menten kinetics, and so values of K'_M may be underestimated. Accurate estimation of K_d with method II requires a significant difference between the concentrations of dimer and total enzyme so that the concentration of monomers can be reliably measured. The total enzyme concentration was adjusted to give measurable concentrations of dimer and monomer. Similar values of K_d were then obtained for a given dimer at a range of total enzyme concentrations. This is more accurate than estimating K_d from the value of K'_M for tyrosine.

Values of K_d were determined with each approach, and the results agree quite well (Table II). Method I gives lower values than method II when K'_M for tyrosine is relatively low. This is due to stabilization of dimers by ATP.

Physical Basis of the Kinetics of TyrTS Mutants. In order to understand the physical basis of the kinetics, the derivations were used to predict the tyrosine dependence of pyrophosphate exchange by TyrTS(Asp-164) at pH 7.8 (Figures 3 and 4). Increasing substrate concentration decreases $[E_m]$ and has two opposing effects on rate of reaction, increasing $\sum[E_\alpha \cdot E_\beta]$ and increasing K'_M. The net result of these changes is seen in v:

$$v = V_{max}[E]_t[T]/(K'_M + [T]) \quad \text{(A29)}$$

$$= V_{max}\sum[E_\alpha \cdot E_\beta][T]/(K_M + [T]) \quad \text{(A30)}$$

Expression A30 was used to predict rates of reaction that were plotted along with experimental data as $v/[E]_t$ against $v/[T][E]_t$ (Figure 4). The theoretical lines are gentle curves that became steeper as they reach the $v/[E]_t$ axis.

K_d for TyrTS(Asp-164) at pH 7.8 is about 120 μM (Table II), which gives predicted data agreeing very well with the experimental points (Figure 4). This agreement is not seen when the value of K_d is changed by a factor of 2 (Figure 4), showing that the predictions are sensitive to K_d. Similar agreement between observed and predicted data was found for dependence upon tyrosine or ATP for the other enzymes at each pH. The method of kinetic analysis assumes that liganded monomeric enzyme does not accumulate (see above). The approach accurately predicts the observed kinetics of TyrTS mutants; therefore, this condition must hold.

The concave curve of the Eadie plot (Figure 4) is similar to that for enzymes that display negative cooperativity. This is because higher concentrations of substrate increasingly favor dimerization, lowering the proportion of monomer so that dissociation of dimer is increased. The value of K'_M is therefore elevated when [S] is raised (Figure 3).

Observed rates of reaction at low tyrosine concentrations (represented by the symbol ○ on Figure 4) are significantly lower than those predicted with the correct value of K_d if assays are commenced by adding enzyme directly to radioactive pyrophosphate together with tyrosine and ATP. This is caused by a lag before stabilization of dimers by the substrates reaches equilibrium. This effect explains the unusual Eadie plot previously reported for TyrTS(Asp-164) (Jones et al., 1985) and was seen for each mutant enzyme, but only at low concentrations of tyrosine and ATP, since the dimers are stabilized rapidly at high concentrations of substrate. This observation was also made during study of dependence upon ATP, demonstrating that monomeric enzymes have low affinities for both

substrates. The artifact is not seen if enzyme is equilibrated with substrates before commencing the assay by addition of radioactive pyrophosphate. Slow dimerization also limits rates of formation of enzyme-bound tyrosyl adenylate during active site titration, giving slow time courses for mutants at position 164 relative to wild-type enzyme (Jones et al., 1985). This also explains the low stoichiometries of active site titrations for these enzymes (Ward et al., 1986), because the final value is a steady state in which rates of formation and breakdown of enzyme-bound tyrosyl adenylate are equal. Slow dimerization would not produce these artifactual results if sufficiently high concentrations of enzyme or substrate are used, but such concentrations would be in excess of practical limitations.

Discussion. Many enzymes are formed by reversible association of inactive subunits to produce oligomers that would obey Michaelis–Menten kinetics under nondissociating conditions [see Kurganov (1981)]. The general method of analysis that is presented here can be modified to apply to all of these enzymes. Some wild-type aminoacyl-tRNA synthetases behave in this way [for example, prolyl-tRNA synthetase from *E. coli* (Lee & Muench, 1969), tryptophanyl-tRNA synthetase from beef pancreas (Iborra et al., 1973), and methionyl-tRNA synthetase from wheat (Chazal et al., 1977)]. Experimental data demonstrate that our method accurately predicts kinetic behavior in such a system, validating the assumptions that were made for analysis of TyrTS mutants. Apparent K'_M increases with increasing K_d (Tables I and II), and the analysis predicts that it would decrease with increasing $[E]_t$. The value of k_{cat} is not dependent upon K_d, because it is achieved only when all of the enzyme is in the dimeric form (Table I). This method of analysis has allowed study of the effects of introducing different side chains into the hydrophobic subunit interface of TyrTS.

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